Microbial Commercial Activity Notice (MCAN):

Contained Use of a Genetically Modified Microorganism

Trichoderma reesei

for the Biosynthesis of an Enhanced Cellulolytic Protein Preparation

TS MC11BM

Novozymes North America, Inc.
John Carroll
3 August 2011

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CERTIFICATION STATEMENT

I certify that to the best of my knowledge and belief:

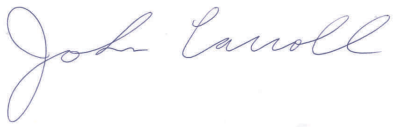
1. The company named in this submission intends to manufacture, import, or process for a commercial purpose, other than in small quantities solely for research and development, the microorganism identified in this submission.

2. All information provided in this submission is complete and truthful as of the date of submission.

3. I am including with this submission all test data in my possession or control and a description of all other data known to or reasonably ascertained by me as required by 40 CFR 725.160.

4. The company identified in this notice has remitted the fee ($2,500.00) specified in 40 CFR 700.45(b). (Pay.gov Tracking ID: 2542I75B; Agency Tracking ID: 74225097900)

Signature of authorized official:



Date: 08/03/2011

_____

John Carroll, Staff Specialist
Novozymes North America, Inc.

[redacted]

CONFIDENTIAL BUSINESS CLAIMS

A limited amount of information submitted in this MCAN is claimed as Confidential Business Information (CBI). The CBI has been limited to the following two categories of information:

Codes used in text margins to identify CBI

1.	Microorganism Identity and Construction	**M.I.**
2.	Production Volume	**P.V.**
3.	Production Information	**P.I.**

Highlighted **M.I., P.V., and P.I.** sections are identified by yellow.

SUBSTANTIATION: **1. Microorganism Identity and Construction** **M.I.**

Substantiation of the combined CBI information, that is marked '**M.I.**' in the text, is given in the following, addressing both the general and the specific questions as required.

(c)(1). For what period of time is a claim of confidentiality being asserted? If the claim is to extend until a certain event or point in time, indicate that event or time period. Explain why the information should remain confidential until such point.

The information is claimed CBI for an unlimited period of time. The information, marked '**M.I**.' in the text, is claimed as Confidential Business Information because it concerns details regarding the rDNA construction of the production strain and, therefore, represents a core technology base of Novozymes. While individual steps in the rDNA construction might be well known or publicly available information, the combination of steps constitutes the information that is claimed as CBI.

(c)(2). Briefly describe any physical or procedural restrictions within the company or institution relating to the use and storage of the information claimed as confidential. What other steps, if any, apply to use or further disclosure of the information?

The information is designated confidential and is only distributed internally to persons who have a documented need in order for them to perform appropriate risk assessment or to obtain necessary approvals by authorities.

(c)(3). Has the information claimed as confidential been disclosed to individuals outside of the company or institution? Will it be disclosed to such persons in the future? If so, what restrictions, if any, apply to use or further disclosure of the information?

The information will only be disclosed to individuals outside the company if this is a necessary part of obtaining approvals by authorities or business agreements. If the information is to be disclosed to such persons, it will only be done under a confidentiality claim or a secrecy agreement.

(c)(4). Does the information claimed as confidential appear, or is it referred to, in any of the following questions? If the answer is yes to any of these questions, indicate where the information appears and explain why it should nonetheless be treated as confidential.xz

[redacted]

(i) Advertising or promotional materials for the microorganism or the resulting end product?
(ii) Material safety data sheets or other similar materials for the microorganism or the resulting end product?
(iii) Professional or trade publications?
(iv) Any other media available to the public or to competitors?
(v) Patents?
(vi) Local, State, or Federal agency public files?

The production organism has been patented/patent pending.

(c)(5). Has EPA, another Federal agency, a Federal court, or a State made any confidentiality determination regarding the information claimed as confidential? If so, provide copies of such determinations.

No.

(c)(6). For each type of information claimed confidential, describe the harm to the company's or institution's competitive position that would result if this information were disclosed. Why would this harm be substantial? How could a competitor use such information? What is the causal connection between the disclosure and harm?

The information represents the state-of-the-art of one of Novozymes' core technologies that has been obtained as a result of substantial investments in research and development within rDNA technology.

(c)(7). If EPA disclosed to the public the information claimed as confidential, how difficult would it be for the competitor to enter the market for the resulting product? Consider such constraints as capital and marketing cost, specialized technical expertise, or unusual processes.

A competitor, already using rDNA technology, would be significantly eased in constructing a similar production strain. It is anticipated that commercialisation would be fairly easy as the proteins produced by the production strain are similar to the protein products currently manufactured and marketed by our competitors.

(d)(1). Has the microorganism or method of production been patented in the U.S. or elsewhere? If so, why is confidentiality necessary?

The microorganism that is the subject of this notice has not been patented. A component used in the construction of the microorganism, however, has a patent pending (see (c)(4) above)).

(d)(2). Does the microorganism leave the site of production or testing in a form which is accessible to the public or to competitors? What is the cost to a competitor, in time and money, to develop appropriate use conditions? What factors facilitate or impede product analysis?

The microorganism is removed from the final protein product.

(d)(3). For each additional type of information claimed as confidential, explain what harm would result from disclosure of each type of information if the identity of the microorganism were to remain confidential.

[redacted]

Not applicable.

(e). Health and safety studies of microorganisms.

The safety studies are claimed as Confidential Business Information because they contain proprietary safety data. The information contained in the studies could be used by a competitor to assist them in constructing microorganisms to produce enhanced biomass conversion preparations and establishing safety based on information for which Novozymes expended significant cost and resources.

SUBSTANTIATION: 2. Production Volume P.V.

The information, marked '**P.V.**' in the text, is claimed as Confidential Business Information because it concerns details regarding the estimated yearly production with the rDNA microorganism.

Substantiation of the information, that is marked '**P.V**.' in the text, is given in the following, addressing both the general and the specific questions as required.

(c)(1). For what period of time is a claim of confidentiality being asserted? If the claim is to extend until a certain event or point in time, indicate that event or time period. Explain why the information should remain confidential until such point.

The information is claimed CBI for an unlimited period of time. Disclosure of this information would harm Novozymes' competitive position (see (c)(6)) below.

(c)(2). Briefly describe any physical or procedural restrictions within the company or institution relating to the use and storage of the information claimed as confidential. What other steps, if any, apply to use or further disclosure of the information?

The information is designated confidential and is only distributed internally to persons who have a documented need.

(c)(3). Has the information claimed as confidential been disclosed to individuals outside of the company or institution? Will it be disclosed to such persons in the future? If so, what restrictions, if any, apply to use or further disclosure of the information?

The information will only be disclosed to individuals outside the company only if there is a necessary part of obtaining approvals by authorities or business agreements. If the information is to be disclosed to such persons, it will only be done under a confidentiality claim or a secrecy agreement.

(c)(4). Does the information claimed as confidential appear, or is it referred to, in any of the following questions? If the answer is yes to any of these questions, indicate where the information appears and explain why it should nonetheless be treated as confidential.

(i) Advertising or promotional materials for the microorganism or the resulting end product?
(ii) Material safety data sheets or other similar materials for the microorganism or the resulting end product?
(iii) Professional or trade publications?
(iv) Any other media available to the public or to competitors?
(v) Patents?

[redacted]

(vi) Local, State, or Federal agency public files?

The information that is marked **'P.V.**' in the text does not appear nor is it referred to in any of the above mentioned documents.

(c)(5). Has EPA, another Federal agency, a Federal court, or a State made any confidentiality determination regarding the information claimed as confidential? If so, provide copies of such determinations.

No.

(c)(6). For each type of information claimed confidential, describe the harm to the company's or institution's competitive position that would result if this information were disclosed. Why would this harm be substantial? How could a competitor use such information? What is the causal connection between the disclosure and harm?

The information concerns details regarding the estimated yearly production of the rDNA microorganism and from this a yearly production of the protein products might be estimated. This information would be valuable to a competitor in calculating and evaluating important key figures, such as production economy and market size.

(c)(7). If EPA disclosed to the public the information claimed as confidential, how difficult would it be for the competitor to enter the market for the resulting product? Consider such constraints as capital and marketing cost, specialized technical expertise, or unusual processes.

See (c)(6) above.

(d)(1). Has the microorganism or method of production been patented in the U.S. or elsewhere? If so, why is confidentiality necessary?

Yes, the microorganism has been patented.

(d)(2). Does the microorganism leave the site of production or testing in a form which is accessible to the public or to competitors? What is the cost to a competitor, in time and money, to develop appropriate use conditions? What factors facilitate or impede product analysis?

The microorganism is removed from the final protein product.

(d)(3). For each additional type of information claimed as confidential, explain what harm would result from disclosure of each type of information if the identity of the microorganism were to remain confidential.

Not applicable.

(e). Health and safety studies of microorganisms.

The safety studies are claimed as Confidential Business Information because they contain proprietary safety data. The information contained in the studies could be used by a competitor to assist them in

[redacted]

constructing microorganisms to produce enhanced biomass conversion preparations and establishing safety based on information for which Novozymes expended significant cost and resources.

SUBSTANTIATION **3. Production Information** **P.I.**

The information, marked '**P.I.'** in the text, is claimed as Confidential Business Information because it concerns details regarding the production information directly related to the process description.

Substantiation of the information, that is marked **P.I.** in the text, is given in the following, addressing both the general and the specific questions as required.

(c)(1). For what period of time is a claim of confidentiality being asserted? If the claim is to extend until a certain event or point in time, indicate that event or time period. Explain why the information should remain confidential until such point.

The information is claimed CBI for an unlimited period of time. Disclosure of this information would harm Novozymes' competitive position (see (c)(6)).

(c)(2). Briefly describe any physical or procedural restrictions within the company or institution relating to the use and storage of the information claimed as confidential. What other steps, if any, apply to use or further disclosure of the information?

The information is designated confidential and is only distributed internally to persons, who have a documented need.

(c)(3). Has the information claimed as confidential been disclosed to individuals outside of the company or institution? Will it be disclosed to such persons in the future? If so, what restrictions, if any, apply to use or further disclosure of the information?

The information will only be disclosed to individuals outside the company, if this is a necessary part of obtaining approvals by authorities or business agreements. If the information is to be disclosed to such persons, it will only be done under a confidentiality claim or a secrecy agreement.

(c)(4). Does the information claimed as confidential appear, or is it referred to, in any of the following questions? If the answer is yes to any of these questions, indicate where the information appears and explain why it should nonetheless be treated as confidential.

(i) Advertising or promotional materials for the microorganism or the resulting end product?
(ii) Material safety data sheets or other similar materials for the microorganism or the resulting end product?
(iii) Professional or trade publications?
(iv) Any other media available to the public or to competitors?
(v) Patents?
(vi) Local, State, or Federal agency public files?

The information, that is marked P.I. in the text, does not appear nor is it referred to in any of the above mentioned documents.

[redacted]

(c)(5) Has EPA, another Federal agency, a Federal court, or a State made any confidentiality determination regarding the information claimed as confidential? If so, provide copies of such determinations.

No.

(c)(6) For each type of information claimed confidential, describe the harm to the company's or institution's competitive position that would result if this information were disclosed. Why would this harm be substantial? How could a competitor use such information? What is the causal connection between the disclosure and harm?

The information concerns details regarding the production process. This information would be valuable to a competitor in evaluating important key processes including, but not limited to, confidential fermentation and recovery operations.

(c)(7) If EPA disclosed to the public the information claimed as confidential, how difficult would it be for the competitor to enter the market for the resulting product? Consider such constraints as capital and marketing cost, specialized technical expertise, or unusual processes.

See (c)(6).

(d)(1) Has the microorganism or method of production been patented in the U.S. or elsewhere? If so, why is confidentiality necessary?

Yes, the microorganism has been patented/patent pending.

(d)(2) Does the microorganism leave the site of production or testing in a form which is accessible to the public or to competitors? What is the cost to a competitor, in time and money, to develop appropriate use conditions? What factors facilitate or impede product analysis?

The microorganism is removed from the final enzyme product.

(d)(3) For each additional type of information claimed as confidential, explain what harm would result from disclosure of each type of information if the identity of the microorganism were to remain confidential.

Not applicable.

(e) Health and safety studies of microorganisms.

The safety studies are claimed as Confidential Business Information because they contain proprietary safety data. The information contained in the studies could be used by a competitor to assist them in constructing microorganisms to produce enhanced biomass conversion preparations and establishing safety based on information for which Novozymes expended significant cost and resources.

[redacted]

SUBMITTER IDENTIFICATION

Submitter

The company which is submitting the Microbial Commercial Activities Notification (MCAN) manufactures biotechnologically derived products. The submitter of this MCAN is:

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[redacted]

I. INTRODUCTION

The fungus *Trichoderma reesei* production strain [ ] contains genes from other fungi, *i.e.*, [ ] and [ ], to produce an enhanced cellulolytic protein preparation to be used for ethanol production. These proteins, in addition to the proteins produced by the *Trichoderma reesei* host itself, are used primarily in biomass conversion of cellulosic material to glucose, cellobiose and higher cello-oligosaccharides. **M.I.**

II. MICROORGANISM IDENTITY INFORMATION

1. Recipient Microorganism (Host Strain)

1.1 Taxonomy

The host strain, [ ], is *Trichoderma reesei* derived from strain [ ]. **M.I.**

The taxonomic characteristics of the host strain of *Trichoderma reesei* [ ], are the following:

Name:	*Trichoderma reesei*
Class:	Sordariomycetes
Order:	Hypocreales
Genus:	*Trichoderma*
Species:	*reesei*

Reference: Kuhls K., Lieckfeldt E., Samuels G.J., Kovacs, Meyer W., Petrini O., Gams W., Borner T. & Kubicek C.P. 1996. Molecular evidence that the asexual industrial fungus *Trichoderma reesei* is a clonal derivative of the ascomycete *Hypocrea jecorina*. *Proc. Natl. Acad. Sci. U.S.A.* 93 (15): 7755-7760 (1).

1.2 Pathogenic and Physiological Traits

Trichoderma reesei is a filamentous fungus that reproduces strictly asexually (imperfect fungus). On the basis of DNA analysis, *Trichoderma reesei* has been classified as the asexual form of the ascomycete *Hypocrea jecorina* (1).

Trichoderma reesei, H. jecorina or previous names used for the strain have not been listed in Berufgsnossenschaft der Chemischen Industrie Merkblatt B 007/Pilze (nr 8/2002) as being Biological Risk class 2 or higher. It is classified as a Class 1 organism according to the NIH guidelines. Thus, both *Trichoderma* and *Hypocrea* species are regarded as Biological Risk Class 1 organisms and are not considered pathogenic to humans. Extracellular cellulases are produced by many bacteria and fungi normally found in environments rich in decaying plant material. An analytical report on the 'Metabolite potential of *Trichoderma reesei*' concludes that Novozymes' strains of *Trichoderma reesei* do not produce secondary metabolites of toxicological concern. (Appendix A -- Analytic Report, Center for Microbial Biotechnology BioCentrum – DTU, Technical University of Denmark, September 2004).

1.3 Prior Reports of Extended History of Safe Industrial Use

Trichoderma reesei has a long history of safe use in industrial-scale enzyme production (2-5). Historically, cellulases produced by Trichoderma reesei are used in food, animal feed, pharmaceutical, textile, pulp and paper applications (2). According to Hjortkjaer, industrial cellulases produced by fungi including

[redacted]

Trichoderma reesei "[have] a long history of safe use in the production of food and as digestive aids without having given any evidence of possible toxicity" (5, see p. 62). In addition, a pectin lyase enzyme produced by Trichoderma reesei was determined to be a GRAS (Generally Recognized As Safe) substance by qualified experts and has been determined to be safe for food uses (GRAS notice 32 letter, 20- April-2000; GRAS notice 333 letter 24-September-2010 http://www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?rpt=grasListing).

Further, numerous notifications using *Trichoderma reesei* as host organism have been reviewed and found to be acceptable by EPA (see MCANs listed under EPA's Biotechnology Program Under TSCA Notifications, FY98 to Present; www.epa.gov/biotech_rule/pubs/submiss.htm, i.e., J03-0001, J03-0002, J04-0001, J04-0005, J05-0001, J05-0002, and J06-0001, J07-0001, J09-0001, J09-0002, J09-0004, J10-0002).

Also, the current *Trichoderma* host has been used as a non-genetically modified production strain by Novozymes for commercial production of cellulase and hemicellulase products (3) as well as a genetically modified production strain for protein preparations (see J07-0001, J09-0002, J09-0004). Nevalainen *et al.*, concluded that recombinant "techniques have been used to improve the industrial production strains of *Trichoderma reesei* and, in addition, considerable experience of safe use of recombinant *Trichoderma reesei* strains in industrial scale has accumulated. Thus, *Trichoderma reesei* can be generally considered not only a safe production organism of its natural enzymes, but also a safe host for other harmless gene products." (2, abstract).

As stated in section 1.2, an analytical report on the 'Metabolite potential of *Trichoderma. reesei*' concludes that Novozymes' strains of *Trichoderma. reesei* do not produce secondary metabolites of toxicological concern (Appendix A -- Analytic Report, Center for Microbial Biotechnology BioCentrum – DTU, Technical University of Denmark, September 2004).

Based on the foregoing information, it is concluded that the current *Trichoderma reesei* organism can be considered a safe host organism for expressing industrial proteins and can be classified as a non-pathogenic and non-toxigenic microorganism as well

1.4 Development of the Host Strain

M.I.

T. reesei, [ ]was isolated from the wild-type strain, [ ], using [

]

[**M.I.**

M.I.

]

[redacted]

2. Donor Organisms

2.1 Donor organism for the Expressed Genes – [

] **M.I.**

[ ] and [ ] are the Novozymes strains, isolated from soil samples [ ], used as donors.

2.2 Pathogenic & Physiological Traits and Safe Use of Protein

The genetically modified *Trichoderma reesei* strain contains genes from [ ] **M.I.** and [ ]. [ ] is classified as a Risk group 1 organism according to NIH guidelines. This genetically modified organism also contains genes from the Class 2 organism [ ]. [ ]

Genes are not correlated with pathogenicity. The genetically modified organism is classified as a Risk group 1 organism. **M.I.**

2.3 Donor for the promoter

The promoter is from *Trichoderma reesei* strain [ ]. **M.I.**

2.4 Donor for the terminator

The transcriptional terminator is from *Trichoderma reesei* strain [ ]. **M.I.**

3. Recombinant Microorganism – Identification of the Production Strain

The recombinant microorganism is *Trichoderma reesei*]. **M.I.**

4. Construction of Recombinant Microorganism

4.1 Cloning

4.1.1 Cloning of the] gene. **M.I.**

The [

plasmid is shown in Figure 1.

4.1.2 Cloning of the] gene **M.I.**

The [

[redacted]

] A map of this plasmid is shown in figure 2.

4.1.3 Cloning of the [ ] gene **M.I.**

The [

] A map of this plasmid is

4.1.4 Cloning of the [ ] gene **M.I.**

The [

] A map of this plasmid is shown in figure 4.

4.2 Structure of the expression plasmids

4.2.1 The [ ] expression plasmid [ ] consists of the following elements. **M.I.**

[redacted]

Figure 1. Map of [_____] expression plasmid [_____].

[

[**Position**	**Size**	**Element**	**Origin**
(bp)	(bp)		

[redacted]

4.2.2 The [ ]expression plasmid [ ] consists of the following elements. M.I.

]

Figure 2. Map of] expression plasmid [ ]

[

Position	**Size**	**Element**	**Origin**
(bp)	(bp)		

[redacted]

] **M.I.**

4.2.3 The]**expression plasmid** [ ] **consists of the following elements.** **M.I.**

[

]

Figure 3. Map for [ ] expression plasmid [ ]

[redacted]

[

Position (bp)	Size (bp)	Element	Origin

[redacted]

The [ ] expression plasmid [ ] consists of the following elements. **M.I.**

[

Figure 4. Map for [ ] expression plasmid [ ].

[

Position (bp)	**Size** (bp)	**Element**	**Origin**

[redacted]

Position (bp)	Size (bp)	Element	Origin

4.3 Construct Information for the expression plasmids

4.3.1 [ ] Construct **M.I.**

4.3.1.1 Overview

[

]

M.I.

Detailed Description of the Construction for [ ]

M.I

M.I.

[redacted]

M.I.

] **M.I**

4.3.2 [ ] **Construct** **M.I.**

4.3.2.1 Overview **M.I**

[

]

Detailed Description of the Construction for [ ] **M.I**

[

[redacted]

]

4.3.3] **Construct** **M.I.**

4.3.3.1 Overview **M.I**

[

]

Detailed Description of the Construction for[ ] **M.I**

[

]

4.3.4] **Construct** **M.I.**

4.3.4.1 Overview

[

]

4.3.4.2 Detailed Description of the Construction for [ ]

[

[redacted]

]

4.4 Construction of recombinant production organism **M.I.**

[

]

M.I.

4.5 Stability of the Construction and Genetic Transfer Capability

The recombinant production strain is expected to be stable upon prolonged growth. The inserts are chromosomally integrated and, as a result, they are poorly transferred to other organisms.

4.6 Antibiotic Resistance Gene

The strain does not harbor any antibiotic resistance genes [ ] as the] markers of the expression plasmids are removed [ ] Absence of the [ ] was demonstrated by Southern blot analysis. **M.I.**

5. Classification of the Production Strain

As discussed in sections **I.** 1.2 and 1.3, *Trichoderma reesei* is generally regarded as a non-pathogenic fungus that is widely distributed in nature. It is classified as a Class 1 organism according to the NIH guidelines.

The host organism is non-pathogenic and non-toxigenic and the introduced genetic materials do not encode any known harmful or toxic substances. As a result, the genetically modified *T. reesei* production strain [ ] is considered non-pathogenic and non-toxigenic and safe for its intended use. **M.I.**

The production strain complies with the OECD (Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large Scale Practice) microorganisms.

6. Phenotypic and Ecological Characteristics

Trichoderma reesei is widely distributed in nature (see sections II & VII).

[redacted]

III. BYPRODUCTS

Solid and liquid wastes generated from the production of the enhanced cellulolytic protein preparation are sterilized by a combination of filtration and chemical and physical treatments to ensure that viable cells of the production microorganism are not present in the material released to the environment.

IV. TOTAL PRODUCTION VOLUME

The modified microorganism, which is the subject of this MCAN, is used to produce an enhanced cellulolytic protein preparation. The estimated microbial biomass to be produced annually during the first 3 years of production for years 2011-2013 is listed below in Table 1.

Table 1. Estimated Total Microbial Biomass Annual Production for 2011-2013 **P.V.**

V. USE INFORMATION

1. Microbial Substance

Trichoderma reesei, which is the subject of this MCAN, will be used for the biosynthesis of an enhanced cellulolytic protein preparation. The production microorganism will be removed from the commercially sold enzyme product. The biomass produced during the fermentation is removed from the recovery process stream, inactivated, and discarded.

2. Protein Preparation from the Recombinant Strain

The current use of the enhanced cellulolytic protein preparation is primarily intended to be used in biomass conversion of cellulosic material to glucose, cellobiose, and higher cello-oligosaccharides. The enzyme preparation has a pronounced viscosity-reducing effect on soluble cellulosic substrates.

VI. WORKER EXPOSURE AND ENVIRONMENTAL RELEASE

1. Site Identification

The modified *Trichoderma reesei* microorganism will be used in a manufacturing process at the following location:

Novozymes North America, Inc.
77 Perry Chapel Road
Franklinton, North Carolina 27525.

2. Process Description

[redacted]

The manufacturing plant, discussed above in section VI.1., produces other food grade and industrial-grade proteins using processes that have varied only slightly for over 25 years. During this time, the company has observed procedures based on physical and biological containment and appropriate work practices designed to minimize both the environmental and occupational exposure to the microorganisms used in the manufacturing processes.

The manufacturing process that utilizes the microorganism is a two-step process involving fermentation and recovery steps. The fermentation process comprises 3 main operations: 1) laboratory propagation of the culture; 2) seed or inoculum fermentation; and 3) the main fermentation. These process steps and recovery processes are described in more detail below and an schematic outline of a typical fermentation process flow and a recovery process flow are provided in the published literature (Appendix B – Hjort, C.M., 'Production of Food Additives using Filamentous Fungi' in Genetically Engineered Food: Methods and Detection. Edited by Knut J. Heller, Wiley-VCH Verlag GmbH & Co, 2003, p.97).

2.1 Fermentation

The process used to grow the modified organism and produced the proteins of interest is submerged (or deep tank), aerobic, pure-culture fermentation. The process, except for the preparation of the initial culture, is carried out in sealed vessels carefully designed to prevent both the release of the production organism and the entry of other microorganisms.

The modified organism is an aerobe, an organism requiring oxygen for life and growth. In this respect, the modified organism is similar to the majority of microorganisms used by industry to produce proteins including enzymes, antibiotics as well as biopolymers and other substances that are manufactured by fermentation processes. In order to supply the required oxygen and prevent the introduction of other microorganisms, fermenters used for this type of fermentation are closed pressure vessels equipped with top mounted agitators with sterilizable seals where the agitators enter the vessel, (e.g., double mechanical seals), bottom-mounted air sparger rings to supply and disperse sterile air, and cooling/heating coils for temperature control. During the sterilization the vessels are usually pressurized to 1-2 bars and during fermentation, a pressure greater than atmospheric is usually maintained. The seed and main fermenters used in the remaining two steps in the fermentation process are of this type.

The ingredients used to prepare the nutrient solutions (broths) in which the organism is grown include various carbon and nitrogen sources and other inorganic salts and trace metals. These ingredients are of suitable quality, free of harmful or deleterious substances and free of substances that would inhibit microbial growth or production of the desired proteins or polymers. Each batch of each substance is sampled and tested by the Quality Control Department to ensure the product is in conformance with the desired specifications.

2.2 Laboratory Propagation

A pure culture of the modified organism, which has been maintained as a lyophile or by storage under liquid nitrogen or other standard technique, is aseptically transferred to a flask containing a sterile nutrient agar prepared from various approved ingredients discussed above.

The techniques used to transfer the organism are designed to prevent both the introduction of other organisms into the flask and the release of the modified organism.

[redacted]

The organism is grown until the desired colony formation and density have been obtained. During this time period, the rate of colony formation and the growth, size, and the appearance of the colonies are examined to ensure that a pure culture has been obtained.

When the desired growth level has been reached, the colonies are transferred to a second flask by washing the agar with sterile water from the second flask which had been connected to the agar-containing flask prior to preparation of the initial sterile agar to give a closed system. This process yields a culture suspension that will be used in the next process step.

After transfer of this suspension to the next process step, the seed fermentation, the residue in the flask is examined for foreign microorganisms by plating on standard agar and other tests if desired.

2.3 Seed Fermentation

Prior to preparation of the medium, the seed fermenter is cleaned with a caustic solution, rinsed thoroughly with potable water, then steam sterilized and cooled. The culture medium is prepared from ingredients drawn from the above list, mixed thoroughly and sterilized by heating to 121^{o}C.

After the sterile medium is cooled to the desired temperature, the culture suspension obtained from the laboratory step is transferred aseptically to the sterile medium.

In this step, growth of the organism is continued until the desired biomass is obtained. During growth, parameters such as temperature, aeration rate, and pH are monitored and samples are withdrawn periodically and examined microscopically to ensure culture purity.

2.4 Main Fermentation

The main fermenter is prepared by cleaning with a solution of caustic, rinsing with potable water and steam sterilizing while empty. The growth medium is prepared in a mixing tank using potable water and ingredients drawn from the acceptable list of ingredients. The medium is transferred to the main fermenter and sterilized by heating the mixture under pressure to a temperature greater than 121^{o}C.

Nutrient feed solutions, if required, are prepared and sterilized in closed mixing tanks, using approved ingredients. After preparation of the sterile nutrient solutions is completed, the contents of the seed fermenter are aseptically transferred to the main fermenter where growth of the organism continues. The desired proteins are produced by the organism during this step. During this step, sterile nutrient solutions can be added to the fermenter and portions of the medium can be withdrawn from the fermenter.

The growth of the organism and the production of the proteins of interest are closely monitored by continuous measurement of parameters such as temperature, pH, aeration rate, dissolved oxygen, etc, and performing tests and analyses such as protein concentration, culture purity, nutrient levels, etc., on samples that are withdrawn from the fermenter at predetermined levels.

The procedure used to obtain samples from the fermenter is designed to prevent microbial contamination of the fermenter contents and sample, and minimize environmental release of the producing organism as described in the environmental release and disposal section.

[redacted]

The fermentation process is continued until laboratory test data show that the maximum protein production has been obtained or the rate of protein production falls below a predetermined value. When this condition is reached, the fermentation is stopped.

The sparge rate of the fermenter is typically one volume of air/volume of medium/minute. The rate would reach a maximum of [ ] m^3/minute depending on the volume of the fermenter which typically ranges from [ ]m^3. **P.I.** **P.I.**

2.5 Recovery

The recovery process is designed to separate the desired protein from the biomass and to purify, concentrate, and stabilize the protein preparation.

P.I.

[

] Testing for the presence of the production organism is performed on the final concentration protein preparation.

P.I.

[

]

[redacted]

The concentrated protein preparation from the steps described above is formulated at the production facility into commercial protein preparations by adding diluents such as sodium chloride solution, propylene glycol or other suitable substances and, if necessary, additional preservatives such as those mentioned above. During this portion of the process, the enzyme activity of the preparation is standardized to the desired level.

3. Worker Exposure

3.1 Company Overview

The company management is dedicated to providing a safe work place. For over 25 years, workers have safely handled microorganisms at this site. Only non-pathogenic and non-toxigenic microorganisms are used at the plant. Worker exposure to microorganisms and proteins are kept at a minimum. This is accomplished by a broad range of established Standard Operating Procedures including those that ensure minimum exposure to all microorganisms that are or may be used in the production processes.

Novozymes is dedicated to providing a safe work environment. A broad range of actions are in place including those that ensure minimum exposure to all microorganisms that are used in the production processes. These actions include the following:

- Use of nonpathogenic, non-toxicogenic microorganisms in all fermentation processes. As stated in section II of the MCAN, the modified microorganism meets these criteria.
- Design and selection of process equipment that provide a level of containment that is appropriate for the production microorganisms - Good Industrial Large Scale Practice (GILSP).
- A comprehensive, plant-wide safety program.
- Installation of appropriate engineering controls.
- Procedures for routinely monitoring the performance of the installed engineering controls.
- Developing procedures and work practices that minimize exposure.
- Training in the proper use of the process equipment and safe work practice.
- Providing personal protective equipment for use where necessary and training in use of this equipment.
- An employee health education and monitoring program. A full-time occupational health and safety manager and nurse are on site to conduct training, perform exposure monitoring, and monitor these programs.
- Personnel training in safe handling of all chemicals as required by OSHA regulations.
- Additional training required to safely perform special operations.

Due to the nature of the manufacturing process, the possibility of occupational exposure to the modified microorganism that is the subject of this MCAN is limited to personnel involved in or with the early steps in the process; specifically the laboratory propagation and testing, the fermentation steps and the first steps in the recovery process during which the modified microorganism is removed and inactivated. The final product, the commercial protein preparation product, has been processed to remove the entire modified microorganism.

[redacted]

3.2 Containment

Novozymes has performed a careful evaluation of the modified microorganism and the planned manufacturing process using information that has been included in this MCAN and information on the historical use of the host organism (unmodified) as well as other historical information from other, similar fermentation processes (see section 1.c of the MCAN). This evaluation has led to the conclusion that the process will be performed under conditions that, as a minimum, will be equivalent to the containment level recommended in the original 1986 report titled "Recombinant DNA Safety Considerations" published by the Organization of Economic Cooperation and Development (OECD) (Paris). The recommendations set forth by OCED are reinforced by the National Institutes of Health's original Guidelines for Research Involving Recombinant DNA Molecules by the Recombinant DNA Advisory Committee and the Office of Recombinant DNA Activities and subsequent amendments located on its website (http://www4.od.nih.gov/oba/rac/guidelines/guidelines.html). This level of containment and regular sampling ensure that employee exposure and unintentional release to the environment are below the level that could raise safety concerns.

3.3 Engineering Controls

P.I.

The processes described above discuss a sterilizable piping system through which culture liquids are transferred, and the closed processing tanks form a nearly completely closed system. The] are completely enclosed with stainless steel hoods equipped with local exhaust that minimizes occupational exposure to the incidental release of microorganisms during the [ ] steps. The hoods contain curtains or doors that can be opened to check the operation of the [ ] but still allow for containment of any aerosols present. [ ] of a protein preparation as set forth in this MCAN requires between [ ] and operators are present in the area around **P.I.** the [ ] for approximately 20 to 30% of this time, not counting the time required for] during which no protein is present. These [ ]systems and procedures are used for all fermentation processes in which the producing organism is removed by [ ]. Many of the process steps are controlled by computer systems and other automated control systems that are located in a control room that is isolated from the manufacturing area. These systems provide an additional form of engineering control in that the use of these systems decreases direct contact of operating personnel with the products being manufactured. Air monitoring in the area of the [ ] and other process equipment includes sampling of both proteins and production microorganism levels. Sampling is conducted on a daily basis in areas that contain equipment with the potential to generate aerosols such as [ ]. High volume industrial hygiene samplers are used to measure enzyme levels in the air. Samples are collected on [ ] and are analyzed through the use of activity-based or ELISA methods. Airborne enzyme results are compared against published exposure limits and internal standards to prevent the sensitization and possible development of allergy to the enzyme proteins. Samples that exceed the internal exposure limit of [ ] in follow-up measures which include cleaning, additional air monitoring, and the use of personal protective equipment such as respirators until clearance samples have been obtained.

3.4 Procedure and Work Practices

Effective control of occupational exposure is a goal that requires the active participation of all company personnel in the development and daily use of a combination of appropriate operating procedures and

[redacted]

work practices, and the use of appropriate personnel protective equipment where such equipment is required.

To help achieve this goal, production departments conduct routine safety inspections of their work areas, perform monthly training and hold departmental safety meetings involving both supervisory personnel and operators. A larger cross-functional safety committee meets monthly to review accidents and near-misses as well as topics that affect the larger organization. Training is held regularly for current employees and to train new employees. In addition, another committee oversees conformance with current Good Manufacturing Practices (cGMP) regulations. The committee meets generally once of a month to review conformance with these regulations and discuss any actions needed in this area.

3.5 Health Program

Occupational health and wellness is an important part of the company's philosophy. Annual health screenings are conducted for all production, laboratory, and maintenance personnel. These examinations include audiometric testing, pulmonary function testing, respiratory fit testing and a blood sample test to determine the levels of antibodies to the specific enzyme proteins handled at the Franklinton, NC location. An annual history form is also completed by each employee as part of the examination. The information obtained from the screening and history is reviewed by the company physician and occupational health nurse. The test information and any comments received from the reviewing physician are reviewed with each employee and a summary of the test results is given to each employee.

The Company also has a global Medical Center headed by an Occupational Physician to provide support and guidance for occupational health. Safety & Health personnel from the North Carolina location interact frequently with their counterparts in other global sites. To work towards the goal of eliminating enzyme allergy in the workplace, Novozymes has implemented a program called Zero Enzyme Allergies (ZEAL) that includes occupational health screening as well as emphasis on engineering controls, work practices, and personal protective equipment (PPE). Performance metrics are reported on and tracked globally.

3.6 Employee Exposure

As mentioned previously, the manufacturing process is performed in a predominantly closed, highly automated system which limits the potential exposure of employees to the modified organism. Table 2 depicts the Average Workers per Facility and Process.

Table 2. Average Workers per Facility and Process **P.I.**

[a] Microbiology personnel provide partial coverage to night shift
[b] Production shifts are 12 hour rotating

[redacted]

4. Information on Release of the Production Organism

An environmental monitoring system is in place to quarterly test for the presence of GMOs in solid and liquid wastestreams. This monitoring system is designed to exceed the compliance requirements as set forth in Part 725 - the TSCA Reporting Requirements and Review Process for Microorganisms. The facility also has control equipment that reduces the population of microoganisms in air emissions.

5. Transport of the Production Organism

As indicated previously, the production organism will be used for production of cellulose-degrading protein preparations at only one site in the U.S. and the production microorganism is removed from the commercial product. 4. In general, the production organism will not be transported unless for disposal purposes. Treated wastewater, which is in compliance with the regulations as discussed in section VI.4, may occasionally be transported to municipal wastewater treatment plants.

6. Process Waste Disposal

6.1 Permits

Typically, Novozymes sprays treated wastewater on privately-owned land. The company also discharges a portion of treated wastewater to a publicly-owned waste treatment plant under permit. The biomass produced during fermentation is an intermediate product that is removed from the process stream, inactivated, and discarded as described below. The use of the inactivated biomass as fertilizer is licensed by the North Carolina State Department of Agriculture.

The current permits are: **P.I.**

[

]

P.I.

The recovery process at Novozymes described above generates a "solid" waste, an [ ] and spent biomass from the] and other [ ] employing [ ], and a liquid waste from the] and the wash water used in equipment cleaning operations. Disposal of these process wastes are carried out as described below.

6.2 Solid Wastes

P.I.

[

[redacted]

] At the end of each [
] and] is continued until the material in the trough has been diluted substantially, as indicated by [ ]. The remaining contents of the trough are transferred to the sludge storage tanks. [

. The storage tanks are sampled and tested quarterly to ensure that the slurry does not contain any viable production organism. A portion of the combined biomass is dewatered through a decanter centrifuge. The concentrate from the centrifuge is routed to the wastewater treatment system. Calcium hydroxide is blended with the cake as it drops from the centrifuge to raise the pH above 12.0. The cake is [
].

The company also has the capability under permit to land-fill or land-apply the cake as a dry material. Final disposal is by land application to approximately [ ] acres of agricultural fields owned by the company and neighboring landowners. The diluted sludge is surface applied to the soil. The nitrogen and other plant nutrients in the waste are used as fertilizer. This disposal operation is carried out under permit [ ], granted by the State of North Carolina Department of Environment and Natural Resources, under the applicable EPA regulations governing the disposal of solid wastes. It is also a registered fertilizer by the North Carolina Department of Agriculture. This method of solid waste disposal has been used for 26 years.

Occasionally] need to be placed in a landfill. In this circumstance, these [ ] are first treated with caustic (potassium hydroxide) and then sent to a municipal solid waster type landfill (Brunswick Waste Management Landfill, 107 Mallard Crossing Rd., Lawrenceville, VA 23868). GMM testing is conducted prior to any release of [ ] to the landfill.

6.3 Liquid wastes

P.I.

Liquid waste is generated in the form of wastewater from the [
], and spent wash water and cleaning solutions from the process tanks and equipment. The cleaning operations are conducted with [
] The production organism in the collection sump. The wastewater in the sump is pumped through a primary clarifier to an activated sludge treatment system owned and operated by Novozymes and located on Novozymes property. The activated sludge system is designed to reduce the organic carbon and nitrogen levels in the wastewater, and to prevent emission of odors. A final clarifier separates wastewater biomass from liquid by gravity separation. The primary clarifier and waste activated sludges are combined with the spent biomass at the buffer tank.

P.I.

The treated wastewater is collected in two lagoons for subsequent land application by spray irrigation,

[redacted]

under permit [ ] granted by the State of North Carolina Department of Environment and Natural Resources, under the applicable EPA regulations governing the disposal of wastewater. The Company also discharges a portion of the wastewater to local municipal wastewater treatment systems through a pipeline. In addition, a "pump and hall" permit can be obtained, if needed, which is granted with the permission of the municipality and the State of North Carolina Department of Environment and Natural Resources, under the applicable EPA regulations governing the disposal of wastewater. The lagoons are sampled quarterly and tested for the presence of the organisms used for enzyme production.

Novozymes discharges non-production waste (domestic sewage) directly to the Franklin County Wastewater Treatment Plant, and Discharges cooling tower water to surface water through permit]. Both of these waste water streams have been determined not to contain GMM based on sampling and our process knowledge.

6.4 Exhaust Air

P.I.

The production organism requires [ ]. Exhaust air from all fermenters is collected in a plenum which is kept under a slight negative pressure. This air stream passes through a cyclone separator designed to remove entrained droplets and mist. Any collected liquid and solids is sent to the wastewater treatment system or spent biomass treatment system described above. The off-gas is then discharged to the atmosphere through the fermenter exhaust stack. [

Although, air sampling was conducted in the past, the company has instituted a worldwide initiative to conduct soil sampling in lieu of direct air sampling. This policy change is a result of a 1998 EU Directive (No. 98/81, October 26, 1998) and an agreement between the Danish authorities and Novozymes that monitoring soil samples for growth of GMMs is a better way of determining environmental impact than actual monitoring air samples. As a result, Novozymes developed a worldwide soil/vegetation sampling program. Soil/vegetation sampling is viewed as a better means of controlling emissions and is a more reliable measurement of the impact of GMMs on the environment than air sampling.

P.I.

The exhaust air from the [ ] is vented to the roof of the production facility.

VII. HEALTH AND ENVIRONMENTAL EFFECTS DATA

1. Safety Studies

[

]

[redacted]

Below find the identification for each study type and a summary of conclusions from each study. For the full study reports see the Appendices as indicated at the end of each study conclusion remarks.

1.1 Toxicity Study by Oral Gavage Administration to CD Rats for 28 days

It is concluded that oral administration of [ ]to Crl:CD® (SD) rats at doses up to 10.0 mL/kg/day for 28 days was well tolerated.

See Appendix C for the study report.

1.2 Test for Mutagenic Activity (Ames test)

It was concluded, that the results of the experiments, described in this report give no indication of mutagenic activity of [ ] in the presence or absence of metabolic activation, when tested under the condition employed in the this study.

See Appendix D for the study report.

1.3 Cytotoxicity Test

The results show, that [ ] is non-cytotoxic, in the present in vitro Neutral Red Uptake assay applying ht mouse fibroblast cell line L929 as test system.

See Appendix E for the study report.

1.4 Assessment of Ready Biodegradability

Mean oxygen consumption in biotic mixtures containing [ ] was equivalent to [

]

Substances are considered to be readily biodegradable in this type of test if oxygen consumption is equal to or greater than 60% of the ThOD of the test mixtures within ten days of the consumption achieving 10%. Therefore [ ] was considered to be readily biodegradable under the conditions of this test.

See Appendix F for the study report.

1.5 Acute Toxicity to *Daphnia magna*

Under the conditions of the test,[ ]was not found to be acutely toxic to Daphnia magna [ ]

[redacted]

[

]

See Appendix G for the study report.

1.6 Acute Toxicity to Fish

After 96 hours, no mortality or significant sub-lethal effects on the fish were observed at a [

]

See Appendix H for the study report.

1.7 Algal Growth Inhibition Assay

Under the conditions of the test,[ ]was not found to be acutely toxic to *Pseudokirchneriella subcapitata* [ ]

Consequently, the 72-hour EbC50, ErC50 and EyC50 values for[ ]could not be calculated but must be >100 mg TOS/L and the "no observed effect concentration" was 100 mg TOS/L.7.

See Appendix I for the study report.

2. Environmental Testing and Monitoring Program

Novozymes has established a Genetically Modified Microorganism (GMM) monitoring program including the standardizing operating procedures for the Determination of GMO Environmental Action Levels Using Fermentation Populations, and Detection of Production Strains in Environmental Samples. The GMM monitoring program consists of sampling in four areas: measurement in waste water, biomass, filter pads for landfill (if applicable), and a retrieval program of soil samples. Typically, 2 measurements of bacterial and fungal production strains are tested in waste water, biomass, and filter pads for landfill are tested quarterly. The soil retrieval program is tested one time per year near the end of the growing season at five locations at the NZNA site. The purpose of the soil retrieval program is to see if GMMs are able to establish and survive in the environment outside the closed systems. Based on monitoring samplings, environmental release is negligible.

3. Environmental Programs and Initiatives

State of North Carolina's Environmental Stewardship Initiative Program

Novozymes North America, Inc. was selected as the State of North Carolina's first Environmental Steward in its Environmental Stewardship Initiative program (http://www.p2pays.org/esi/; http://www.p2pays.org/news/press_releases/022703.asp).

[redacted]

4. Conclusions

Along with bacteria and other fungi, *Trichoderma reesei* is widely distributed in nature especially among decaying matter. A non-genetically modified *T. reesei* has been used safely for the production of cellulases at this site since 1995. Based on a review of the literature, *Trichoderma reesei* has not been known to be associated with human or veterinary pathogenicity. The exception would be the possibility of an opportunistic infection in severely immunocomprised or debilitated individuals. Further, there are no known reports to indicate the formation of toxins from this strain of *Trichoderma reesei* (see Appendix A).

Existing classification of *Trichoderma reesei* as a Biological Risk Class I microorganism further substantiates that this microorganism is low risk and is not a potentially dangerous organism.

In conclusion, the production organism is not part of the final protein preparation product sold to customers, the microorganism is handled in a contained environment at the plant site, the microorganism is inactivated prior to release to the environment and subsequently very little, if any, viable micoorganism is expected to enter the environment. Therefore, based on this information, *Trichoderma reesei* does not present an unreasonable risk to the environment and to humans

VIII. LIST OF REFERENCES

(1) Kuhls K, Lieckfeldt E, Samuels GJ, Kovacs, Meyer W, Petrini O, Gams W, Borner T. and Kubicek CP, 1996, 'Molecular evidence that the asexual industrial fungus *Trichoderma reesei* is a clonal derivative of the ascomycete *Hypocrea jecorina*' *Proc. Natl. Acad. Sci. U.S.A*. 93 (15): 7755-7760.

(2) Nevalainen, H, Suominen, P, and Taimisto, K, 1994, 'On the safety of *Trichoderma reesei*' *Journal of Biotechnology* 37(3):193-200.

(3) Olempska-Beer, Z.S., Merker, R.I., Ditto, M.D. & DiNovi, M.J. Food Processing enzymes from recombinant microorganisms – a review. *Reg. Tox & Pharma* 45:144-158, 2006.

(4) Pariza, M.W. and Johnson, E.A. Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century. *Reg. Tox and Pharm* 33: 173-186, 2001.

(5) Hjortkjaer, 1986, 'Summaries of Toxicological Data – Safety Evaluation of Celluclast, and Acid Cellulase derived from *T. reesei*' *Fd Chem Toxic* 24(1):55-63.

(6) C. Yanisch-Perron, J Vieira and J. Messing, 1985, *Gene 33*:103-119.

(7) Providenti, MA, Mautner, SI, Chaudhry, O, Bombardier, M, Scroggins, R, Gregorich, E, and Smith, ML, 2004, 'Determining the environmental fate of a filamentous fungus, *Trichoderma reesei*, in laboratory-contained intact soil-core microcosms using competitive PCR and viability plating' *Can. J. Microbiol* 50:623-631.

[redacted]

IX. APPENDICES

Appendix A. Analytical Report – Metabolite potential of *Trichoderma reesei*
Appendix B. Hjort, C.M., 'Production of Food Additives using Filamentous Fungi' in Genetically Engineered Food: Methods and Detection. Edited by Knut J. Heller, Wiley-VCH Verlag GmbH & Co, 2003, p.97
Appendix C. Toxicity Study by Oral Gavage Administration to CD Rats for 28 days
Appendix D. Test for Mutagenic Activity (Ames test)
Appendix E. Cytotoxicity Test
Appendix F. Assessment of Ready Biodegradability
Appendix G. Acute Toxicity to *Daphnia magna*
Appendix H. Acute Toxicity to Fish
Appendix I. Algal Growth Inhibition Assay